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OOGENESIS IN *DROSOPHILA VIRILIS*. I. INTERACTIONS BETWEEN THE RING CANAL RIMS AND THE NUCLEUS OF THE OOCYTE

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The polytrophic, meroistic ovaries of flies belonging to the genus *Drosophila* are made up of clusters of egg tubes. Each ovariole is composed of an anterior, sausage-shaped germarium and a vitellarium which contains a single-file array of individual egg chambers that are attached to one another by stalks of follicle cells. In the germarium a single, apical cystoblast gives rise to 16 cells by a series of four consecutive mitotic divisions. These 16 cystocytes are connected by intercellular channels that are arranged in a specific pattern: 2 cells have 4 canals each, 2 have 3, 4 have 2, and 8 have 1 canal each. In the posterior region of the germarium individual clusters are surrounded by a single layer of follicle cells. These enveloped 16 cell clusters then enter the vitellarium (King, 1970).

The ring-shaped canals that connect the 16 sister cells of a cluster are of interest because of the invariable relationship that exists between the number of canals and the developmental fate of the cystocytes. While the 16 cells are all descendants of a single cell and presumably contain identical genomes, two cells (the pro-oocytes) enter meiotic prophase, and the others do not. Each of the two pro-oocytes always has four canals. This paper will give an account of oogenesis in *Drosophila virilis*, with specific emphasis on ring canal structure and function.

Materials and Methods

Females of *Drosophila virilis* from the Pasadena wild type strain were reared at room temperature on David's medium (David, 1962) during a natural cycle of light and darkness. All work was done on mated flies that were between 5 and 8 days old. The ovaries were dissected from etherized flies immersed in *Drosophila* saline solution (see King, Rubinson and Smith, 1956, for the recipe). Whole mount preparations were used to determine the ovariole number, stage distribution, chamber dimensions, and the state of the chromatin in the follicle cells, nurse cells, and oocytes during oogenesis. The DNA was visualized using Feulgen staining (see King, Burnett, and Staley, 1957, page 242 for the procedure).

Ovaries that were to be sectioned were dissected from females immersed in the fixative, which contained paraformaldehyde and glutaraldehyde, both at a final concentration of 4% in a 0.2 M cacodylate buffer (Karnovsky, 1965). The ovaries were fixed for two hours at room temperature, passed through several washes of 0.2 M cacodylate buffer containing 10% sucrose to approximate the osmolality of the fixative, and then post-fixed for two hours in aqueous OsO_4 and $K_2Cr_2O_7$ (both at final concentrations of 1%). Maser, Powell and Philpott (1967) have determined osmolality values for various fixative, buffer, salt, and sugar solutions, and

we employed their data to obtain fixatives and rinsing fluids of similar osmolalities. The ovaries were rapidly dehydrated through an ethanol series and transferred to propylene oxide. The ovaries were infiltrated over-night in a 1:1 propylene oxide: plastic mixture, and then they were transferred to pure Epon-Araldite (Mollenhauer, 1964). Polymerization took 72 hours at 60° C.

Serial sections 0.5 to 2 μ thick were cut using an LKB Ultrotome III fitted with a glass knife. Sections were picked-up with hair loops and transferred to water drops on glass slides. A hot plate was used for rapid evaporation and attachment of selections to the slides. Egg chambers in each of the 14 developmental stages described by King, Rubinson and Smith (1956) were serially sectioned (1 μ) and stained with Richardson's technique (Richardson, Jarett and Finke, 1960). These sections were used to characterize intranuclear structures. Sections for general morphological work were cut 2 μ thick and stained with azure B at a variety of pH values (4, for differentiation of DNA and RNA, and 7 or 9, for non-specific, but more intense staining). In addition, selected 1 μ sections were stained with fast green (pH 2) for detection of proteins and by the periodic acid-Schiff (PA/S) method for polysaccharides (see King, 1960, for the rationale and techniques). All light sections were studied using a Wild-Heerbrugg M20 research microscope fitted with a drawing tube. Using the drawing tube, the magnified images of chambers in various stages were traced on Kodak diffusion sheets. These tracings were then stacked on top of one another in proper sequence to gain information concerning three-dimensional interrelations of various structures.

Tracings of this sort were also useful for volume estimates. By placing a tracing over a ruled grid and counting the squares enclosed by a given outlined structure, an estimate of the area of the sectioned object could be obtained. Since the thickness of the sections and the image magnification factors were known, we could calculate the volume of each section, and by adding together the volumes of all the sections through the object, its volume was determined. The data presented in Figure 7 were obtained by these methods.

Tissues used for electron microscopy were fixed and embedded in the same manner as that described for light microscopy. Silver to grey sections were cut with glass knives using the LKB Ultrotome III and picked-up with an LKB section collector. Two types of copper grids were used: slot grids for serial work, and mesh grids for general work. Carbon-coated Formvar specimen support films were prepared according to the procedure of Meek (1970). The tissue was first stained with saturated, aqueous, uranyl acetate followed by lead citrate (Reynolds, 1963). Sections were viewed and photographed with either a Hitachi HS 8 or HU 11A electron microscope. Tracings were made of selected serial electron micrographs and stacked in proper order to obtain 3-dimensional reconstructions of certain organelles.

Observations and Results

The morphology of canal rims

In *Drosophila virilis* the pattern of interconnections among the 16 sister cystocytes (see inset, Fig. 7) is the same as that first described in *Drosophila melanogaster* (Brown and King, 1964). Only two cells in a cluster have four canals, and it is these cells that differentiate into pro-oocytes in the germarium. One of

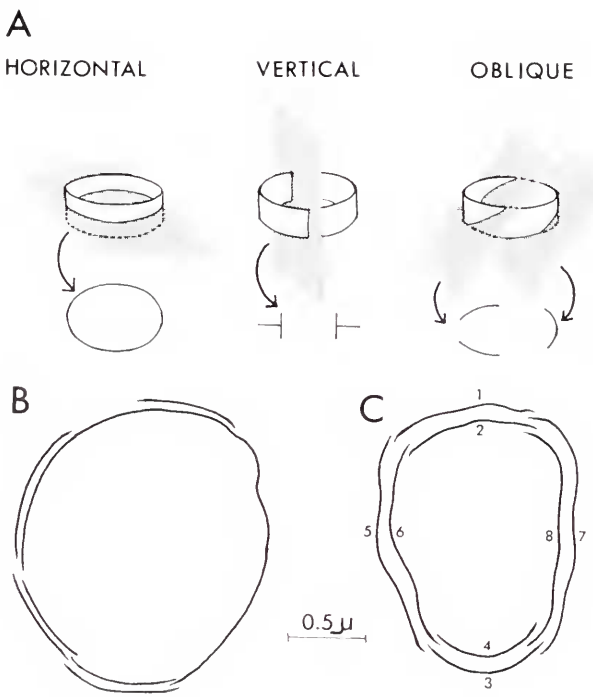


FIGURE 1. (A.) Ring canals as they appear when sectioned in each of three different planes; (B.) a ring canal reconstructed from horizontal sections through a stage 3 oocyte of *Drosophila virilis;* (C.) a ring canal in a germarial cystocyte of *Habrobracon juglandis* (from Cassidy and King, 1969).

the pro-oocytes invariably differentiates into the oocyte, while the other enters the developmental pathway followed by the other 14 nurse cells.

The intercellular connections have been called ring canals because their rims are ring-shaped. A ring has varied appearances when sectioned in different planes, and Figure 1A illustrates the terminology we will use throughout the paper to describe the planes of section and the appearances of the sectioned canals. Ring canals are frequently sectioned in oblique (Figs. 2A, 4D and 5A) and vertical planes (Figs. 2A, 3ABC, and 4A) and rarely in the horizontal plane (Fig. 4C).

When consecutive horizontal sections are photographed, traced, and reconstructed, we find that the canal rim of *Drosophila virilis* is composed of several overlapping leaves (Fig. 1B). The canal rim leaves resemble those described in germarial cystocytes of *Habrobracon juglandis.* In this wasp each rim is composed of 8 leaves arranged in 4 pairs. The opposite leaf pairs have similar dimensions: leaves 1, 2 and 3, 4 are 1.1 μ long and 0.7 μ high; while leaves 5, 6 and 7, 8 are 2.0 μ long and 0.4 μ high (Fig. 1C). The leaves have equal widths. In the *Habrobracon* electron micrographs the space between the paired leaves is wider than in *Drosophila* (compare Figs. 1B and 1C). Unfortunately we do not have all horizontal sections through a *virilis* canal rim and therefore cannot estimate the length and height of each of the leaves. However, we have seen some horizontal

sections that demonstrate that leaves corresponding to 1 and 2 are taller than leaves corresponding to 5 and 6.

In horizontal sections, *Drosophila* canal rims appear to be composed of vertically oriented tubules, each with a diameter of 20 mμ, which are separated from their

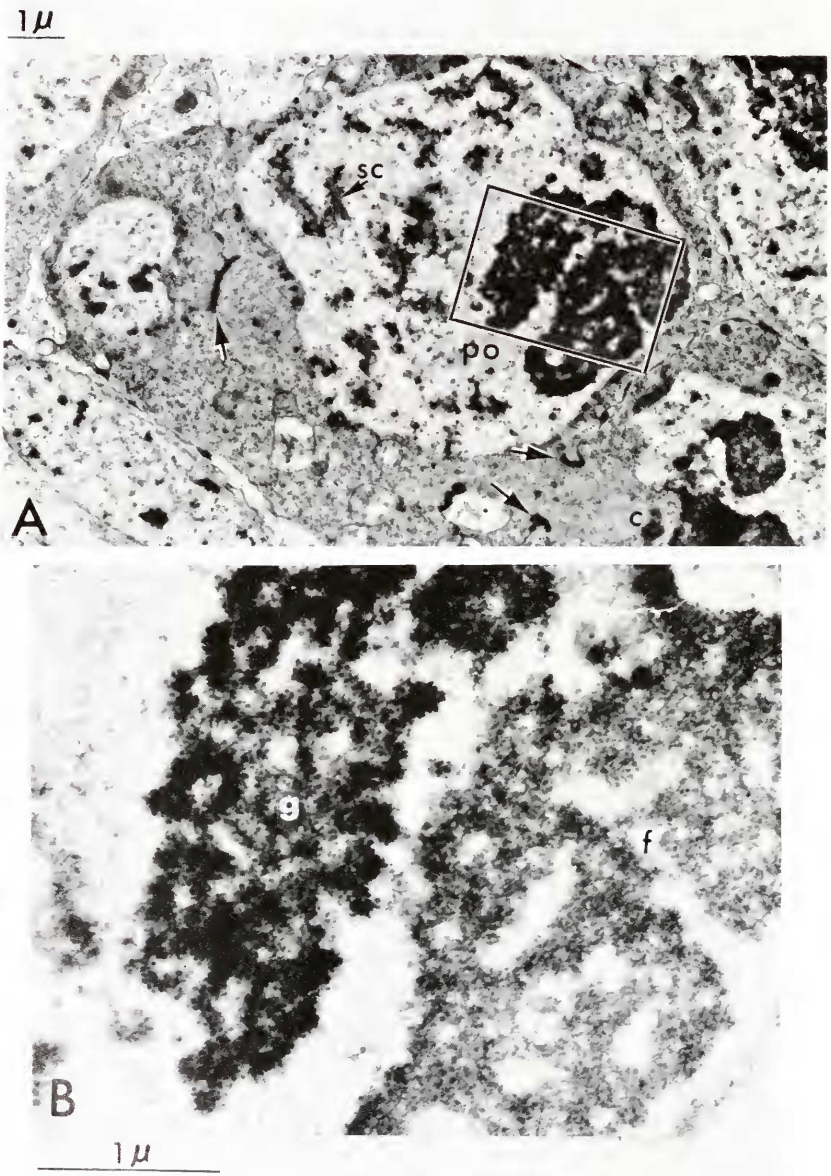


FIGURE 2. (A.) An electron micrograph of a pro-oocyte in the germarium. Its nucleus contains synaptonemal complexes (sc). Arrows point to ring canal rims; one in oblique section, the other in vertical section. A centriole (c) is seen lying near a nurse nucleus; (B.) an enlargement of the nucleolus in A showing both granular (g) and fibrillar (f) elements.

nearest neighbors by approximately 12 mμ. Each canal rim has short pieces of membrane attached to it that radiate out into the cytoplasm. In *Habrobracon*, the dimensions, spacing and orientation of the tubules are similar, and so are the membrane attachments.

Pro-oocytes of *Drosophila virilis* are characterized by nuclei that contain synaptonemal complexes (Fig. 2). The ring canals of pro-oocytes can be classified into four generation groups on the basis of their morphologies and positions. The oldest or first generation canal is always the largest, the most morphologically complex, and the one which connects the pro-oocytes. The diameters of canals from all four generations increase through stage 10B. For example, the first generation canal has a diameter of 1.4 μ in S1 and of 6.8 μ in S10B. The height of the rim also increases with development through at least S8 (from 0.5 μ in S1 to 2.8 μ in S8).

The material located in or adjacent to the canals changes during development. In the most anterior 16 cell clusters, a canal contains flocculent material which assumes an hour glass shape with the waist through the canal. Such areas are free of ribosomes and contain short segments of microtubules, and we assume that these areas represent the remnants of mitotic spindles. By S2 there is a marked decrease in the flocculent material in and adjacent to canals (Fig. 3A). However, short microtubules remain in some of the canals. The younger, fourth generation canal (Fig. 3B) has more tubules and a larger spindle remnant area than the older canal (Fig. 3C). The S2 canals also contain both ribosomes and mitochondria. By S3 no obvious spindle remnants remain in any of the canals (Figs. 4C and 5A). Mitochondria and ribosomes account for the majority of the material seen within canals until the onset of vitellogenesis.

The rims of canals in the youngest 16 cell clusters, found in the anterior half of the germarium (Fig. 2A), are uncoated. However, by stage 1 a coating can be resolved. The first generation canal has the thickest coating, and the fourth generation canal the thinnest. By stage 2, the thickness of the coating increases, but differences are still seen in the development of canals formed at different generations (Fig. 3). The volume of the coating material increases through S6, and then begins to decrease. The thickness of the coating, however, begins to decrease by S5, probably as the result of stretching as the canal expands.

The material coating canal rims has been shown to be a polysaccharide-free protein by cytochemical tests. When the canal is sectioned vertically (Figs. 3B and C), the coating appears to be made up of tightly packed fine particles. However, horizontal sections reveal that the coating contains many microfibrils whose orientation is parallel to the circumference of the canal rim (Figs. 4C and D, 5B). The average diameter of a microfibril is about 65 Å, and the center to center interval between adjacent microfibrils is roughly 160 Å.

Rim material in the cytoplasm

Large clusters of microfibrils are frequently seen free in the ooplasm and occasionally in nurse cytoplasm. The one shown in Figure 5A and 5C is 0.7 μ wide and 3 μ long. Four of these masses have been serially sectioned; the largest one was 0.8 × 6 μ; the smallest, 0.4 × 2.3 μ. They contain microfibrils with diameters in the 65–75 Å range and with center-to-center intervals of about 200 Å. In all masses observed, the microfibrils are oriented parallel to the long axis and

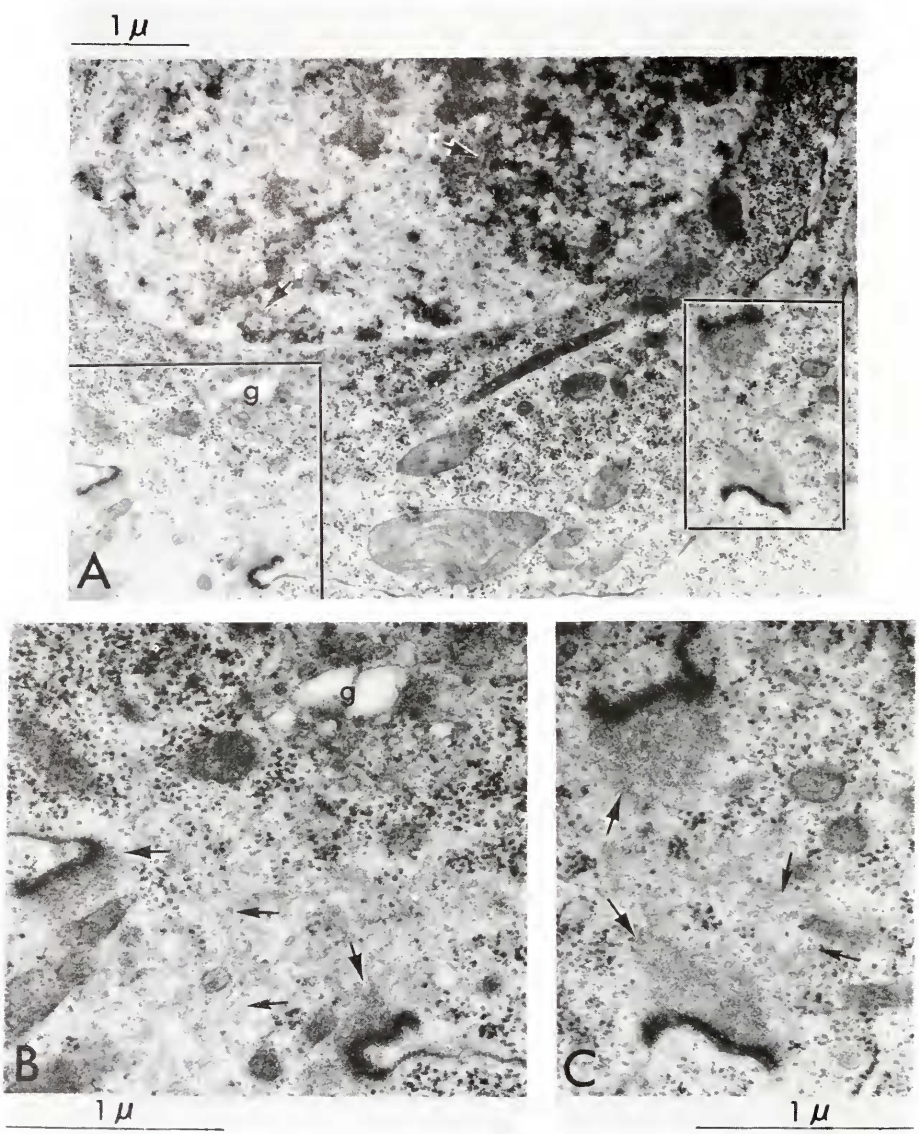


FIGURE 3. (A.) An electron micrograph of a portion of a stage 2 chamber. The nucleus is from a pro-oocyte that has turned to the nurse cell developmental pathway. Note the remnants (r) of old synaptonemal complexes. A well developed golgi apparatus (g) lies next to the nucleus; (B.) an enlargement of the left outlined area in A showing a 4th generation ring canal in vertical section. Large arrows point to the microfibrils coating the rim. The small arrows point to microtubules of the spindle remnant; (C.) an enlargement of the right rectangle in A, showing a 2nd generation ring canal cut in vertical section. Note the increase in the thickness of rim coating and the decrease of spindle remnants relative to B.

perpendicular to the short axis, and they are not enclosed by a membrane. The earliest stage in which these masses have been observed is S3. However, since these clusters are relatively small and are randomly distributed, we may have missed

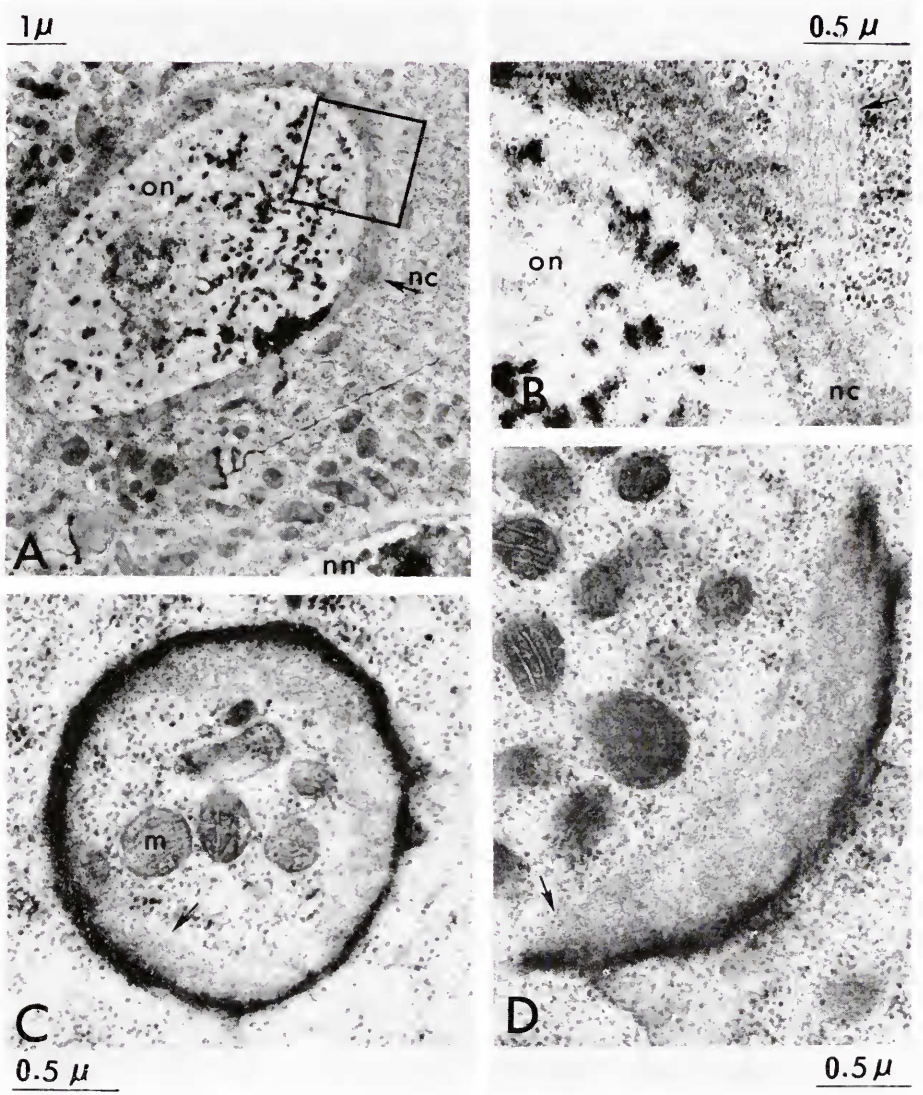


FIGURE 4. (A.) A stage 4 oocyte nucleus (on) and its nuclear coating (nc). The nurse nucleus (nn) has no coating; (B.) an enlargement of the square in A, showing a microfibrillar mass (arrow in contact with the nuclear coat (nc); (C.) a horizontal section through a stage 3 ring canal. The arrow points to microfibrils which are oriented circumferentially about the ring canal rim. The canals contain mitochondria (m) and ribosomes; (D.) an oblique section through a S4 ring canal. The arrow points to microfibrils in the rim coating.

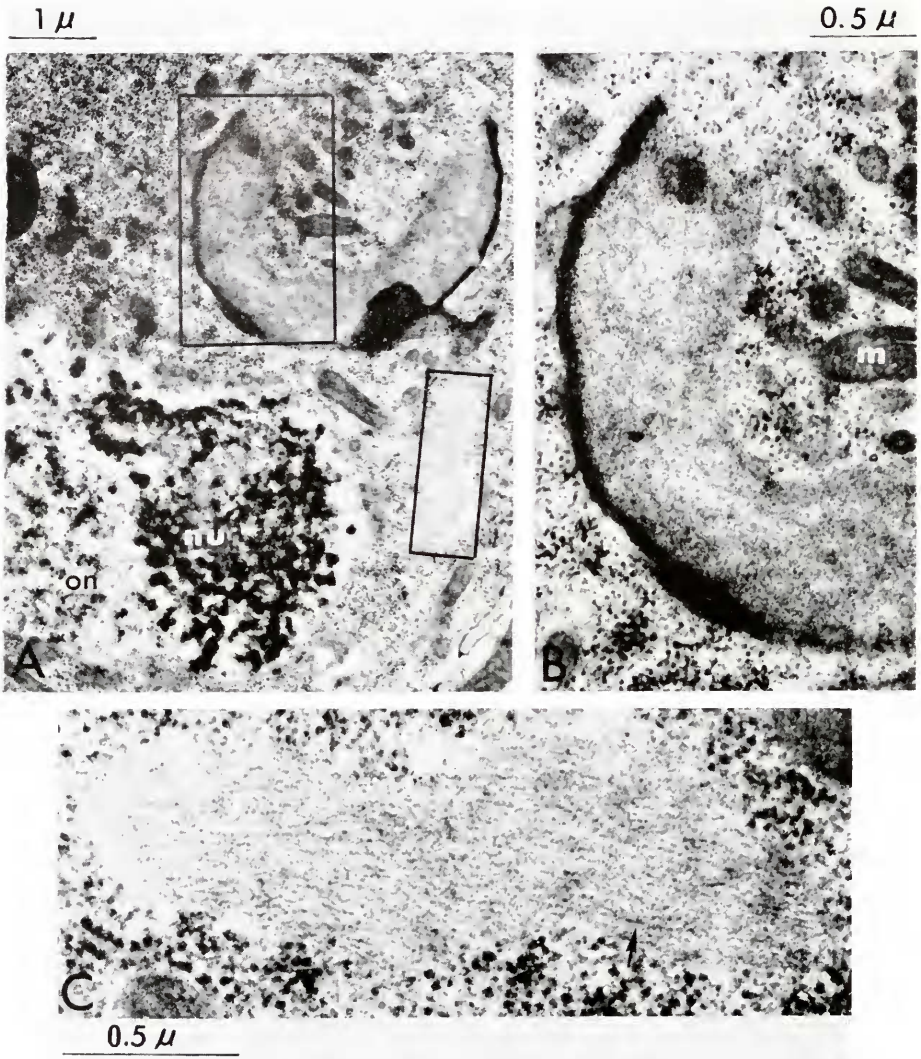


FIGURE 5. (A.) A stage 3 oocyte nucleus (on) containing a fragmenting nucleolus (nu); (B.) an enlargement of the large rectangle in A, showing the rim coating in an obliquely sectioned ring canal; (C.) an enlargement of the small rectangle in A, which shows the fine structure of a cytoplasmic mass of microfibrils. The arrow points to several microfibrils which are oriented parallel to the long axis of the mass.

them in younger cystocytes. Two observations point to canal rims as a source of the clusters of microfibrils seen in the cytoplasm. First, there is similarity of microfibril size and spacing. Secondly, the microfibrils are seen first on canal rims (S1) and later (S3) in cytoplasmic clusters. By S7 the quantity of material on canal rims begins to decrease, and this may be due to the coating material detaching to form the cytoplasmic masses.

The coating of the oocyte nucleus

The fate of these masses is even more intriguing. In one series of electron micrographs, a mass of microfibrils ($0.4 \times 2.5\ \mu$) was seen attached to a coating which is forming about the oocyte nucleus (Figs. 4A and B). From a static picture it is obviously impossible to determine whether the mass is leaving or entering the coating. However, we favor the idea that the microfibrils detach from the rim and attach subsequently to the oocyte nucleus because as is shown in Figure 5A, masses of microfibrils are first seen in the cytoplasm before there is appreciable coating on the oocyte nuclear envelope. The microfibrils around the rims and in the cytoplasm are oriented in parallel while the fibrils in the "amorphous" nuclear coating show no consistent orientation.

The coating or "halo" begins to be formed about the oocyte nucleus in stage 3 (see S3 of Fig. 6). Initially this coating is incomplete, with gaps free from deposits and periodic clumps of the material. By S4 this coating is almost continuous (Fig. 4A). From S5 through S9 the halo is complete, and it continues to accumulate until it reaches a thickness of about 1 μ with a calculated maximum volume of 400 μ^3 (see Fig. 7). We calculated the volume of the halo by subtracting nuclear volume from the volume determined on the basis of nuclear radius plus average halo thickness. After the S9 peak, the character of the coating begins to change. By S10A small gaps reappear (see Fig. 6) with a concurrent decrease in the thickness of the coating. The changes in the character and thickness of the coating may indicate that the addition of new halo material has not kept pace with the rapid growth of the oocyte nucleus. Stage 11 is the last stage before the breakdown of the nuclear envelope in preparation for the 1st meiotic division. The material coating the S11 nucleus is less dense than at the previous stages. In a stage 8 oocyte (Figs. 8A and 8B) the density of the coating is such that mitochondria and ribosomes are excluded from the halo region. However, in stage 11 this exclusion is no longer complete.

One datum worth stressing is that the coating is restricted to the oocyte nucleus (see Fig. 4A). We found no coatings on the nuclei of sister nurse cells, although many were photographed or traced (Table 1) and many more observed. The nurse cell derived from a pro-oocyte also lacks the coating.

The halos are fast green-positive, PA/S negative, Feulgen-negative, and stain green with azure B (pH 4). We conclude that the coating contains protein and lacks appreciable amounts of polysaccharides and nucleic acids. The amorphous appearance of the nuclear coating may indicate that the protein fibers coil up prior to their deposition or that uncoiled microfibrils precipitate on the nucleus in a "brush heap" configuration. During stages S3 through S6 the coating resembles that seen when ring canals are sectioned vertically, exposing the rim coating microfibrils only in cross and tangential sections (Fig. 4A). Later some compacting or clumping of halo material occurs (Figs. 8A and 8B).

Behavior of oocyte nucleus

The cytological changes which characterize the growing oocyte nucleus are diagrammed in Figure 6. Meiotic prophase must begin in the pro-oocytes immediately following the final cystocyte mitosis. The most anterior pro-oocytes we observed were always in a stage where synaptonemal complexes were forming

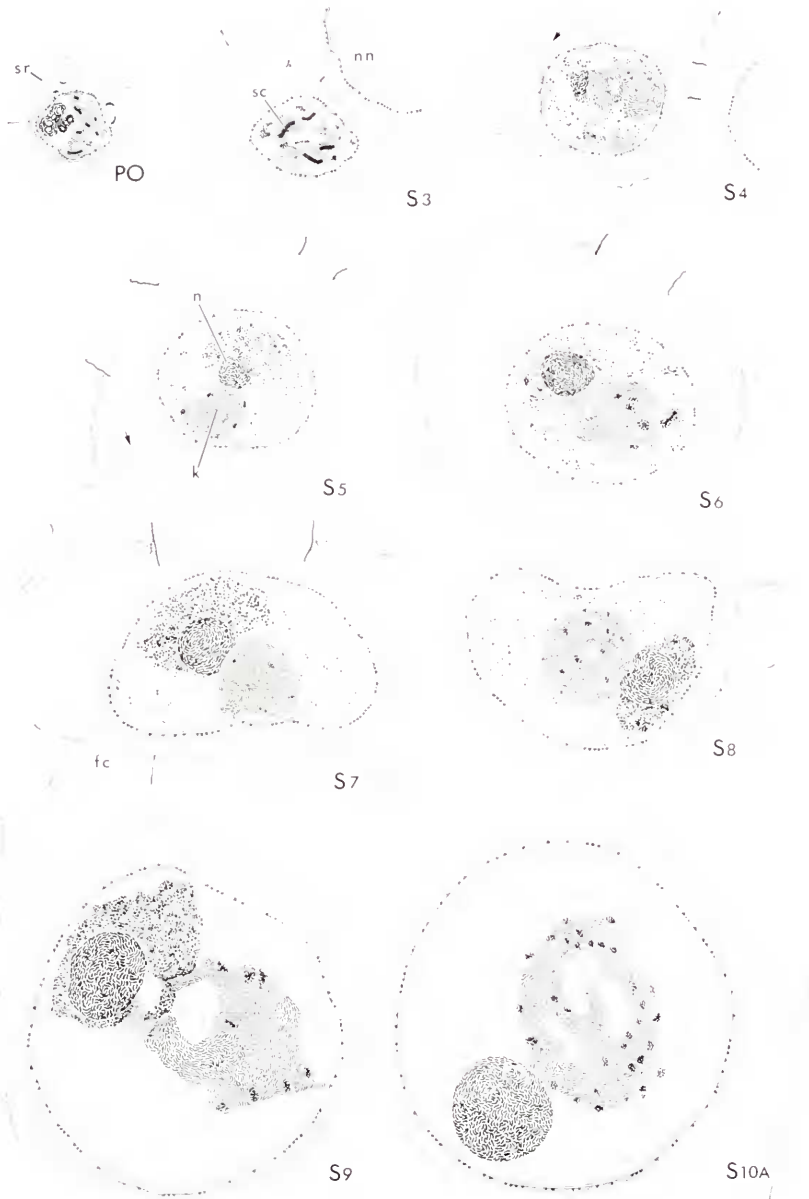
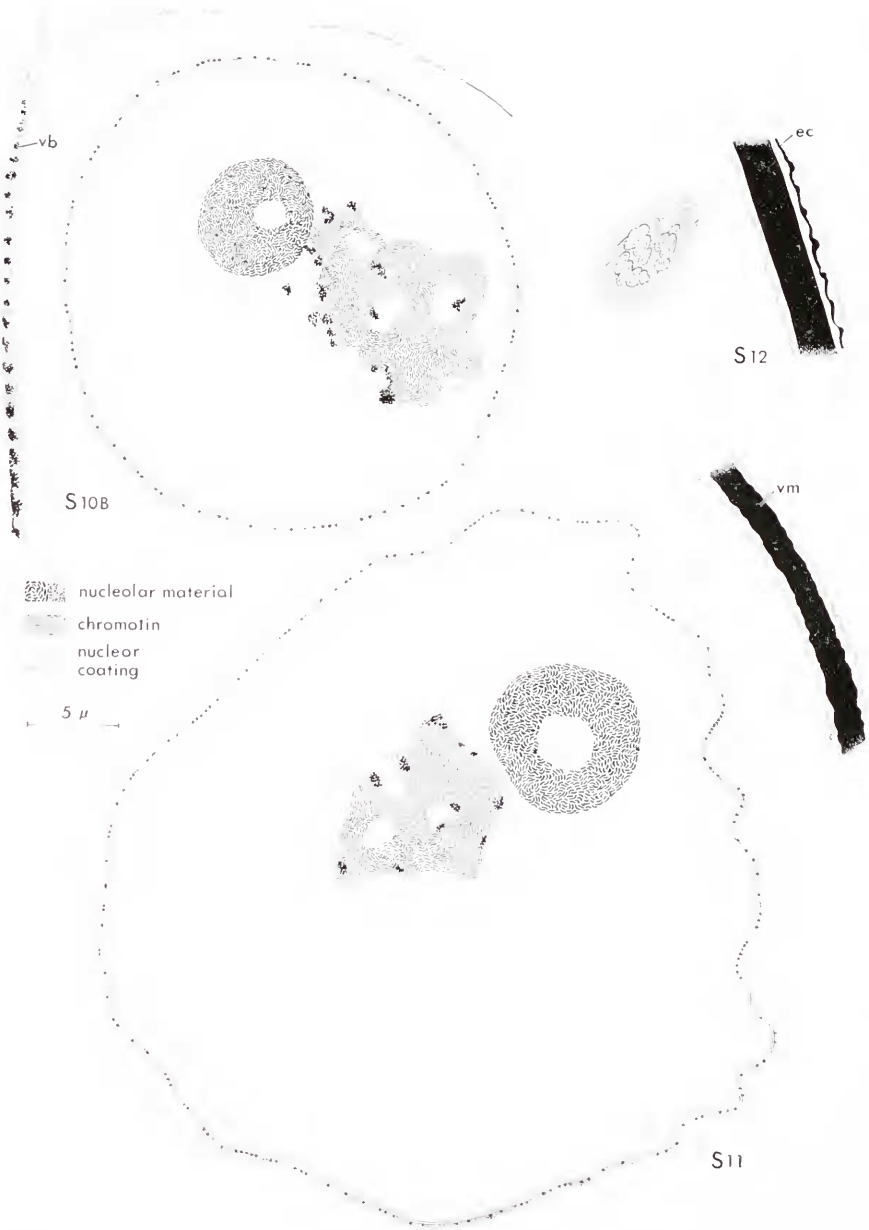


FIGURE 6. Diagrams of stages in the development of the oocyte nucleus from the pro-oocyte (PO) through stage 12. Synaptonemal complexes (sc) occur in the pro-oocyte (PO) and stage 3 (S3) nuclei. Spindle remnants (sr) lie near young ring canals. In oocytes later than S8 ring canals were not included in those sections that contained the nucleus. An amorphous halo forms about the oocyte nucleus in S3 through S9. During S10 and S11 the coatings become thinner and discontinuous. Arrows point to cytoplasmic masses of microfibrils. In S5 a spherical nucleolar structure (n) appears, and it continues to grow through



S11. By S5 the chromatin has aggregated into a karyosome (k) which expands through S10B. In S5, small packets of material (depicted as heavy dots) are seen near the nucleolus, and they persist through S9. Small clumps of granules are associated with the karyosome from S6 through S11. In S10B, vitelline bodies (vb) are deposited, and these coalesce to form a vitelline membrane (vm) by S11. The endochorion (ec), is laid down during S12. The S12 oocyte has distinct chromosomes associated with spindle microtubules. No nuclear envelope or centrioles are present.

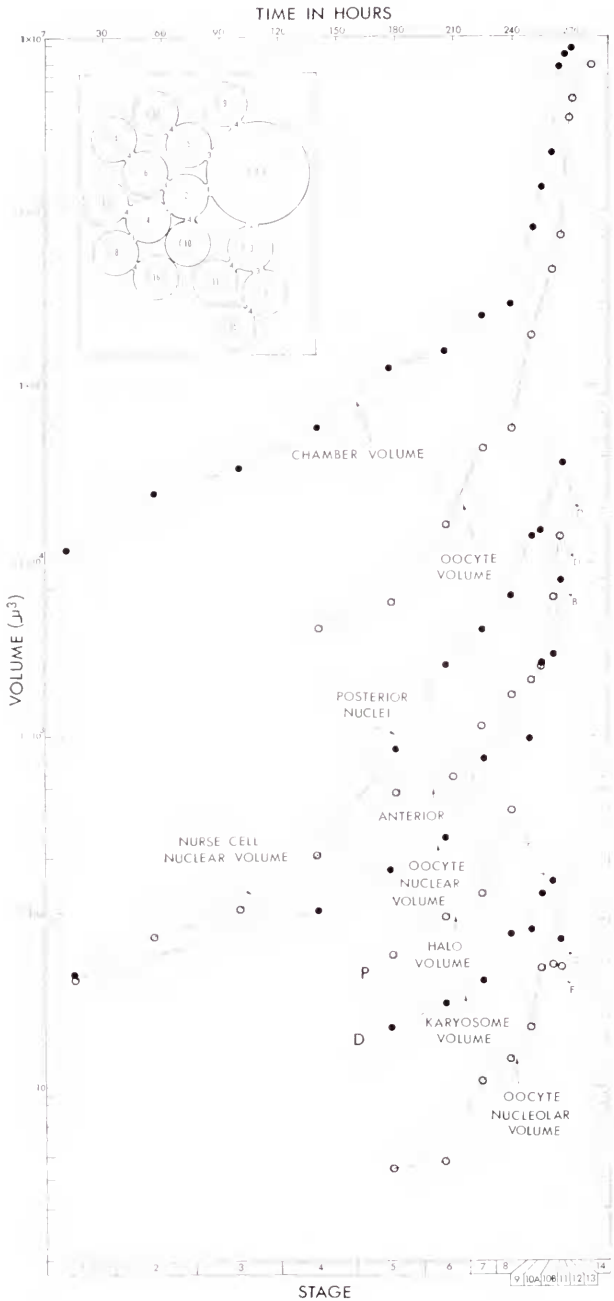


FIGURE 7. The volumes of various ovarian structures as a function of developmental stage; B, breakdown of nuclear envelope; C, condensation of karyosomes into individual bivalents; D, degeneration of nurse cell nuclei; F, fragmentation of the oocyte halo and nucleolus. Inset: the pattern of interconnected fourth generation ovarian cystocytes seen in *Drosophila virilis*.

(zygonema). Leptonema must be very rapid, since it was not observed. In the two germaria we sectioned there were eight 16-cell clusters which were considered newly formed, since they were adjacent to clusters containing 4 or 8 cells. Synaptonemal complexes were never seen in 8-cell clusters; whereas in the adjacent 16-cell clusters the two cells with 4 canals contained synaptonemal complexes. In *D. melanogaster* complexes have been reported to occur in 75% of the newly formed 16 cell clusters (Koch, Smith, and King, 1967).

By the time the oocyte enters the vitellarium it is in the pachytene stage, and synapsis of homologues is complete. Meanwhile, the other pro-oocyte turns to the nurse cell developmental pathway, and the synaptonemal complexes disappear. The true oocyte contains these complexes through S3. In S4 complexes are missing, and chromatin masses are seen distributed throughout the nucleus. By S5 the chromatin is incorporated into a karyosome which expands until S11, when it condenses (see Fig. 7). During S12 the nuclear envelope breaks down, short spindle microtubules appear, and the condensed chromosomes take up positions characteristic of prometaphase. In S13 and 14 the chromosomes are arranged on the metaphase plate, and the spindle microtubules lengthen. We have seen no evidence of either centrioles or asters in our electron micrographs of S12 and S13 meiotic spindles.

In *Drosophila virilis,* from the time the 16 cell cluster forms through S2 or S3, the oocyte nucleolus has granular and fibrillar elements quite similar to those seen in the nucleoli of nurse cells (Figs. 2A and 2B). The nucleolus then begins to fragment. The granular elements are lost, and the fibrillar portions of the old nucleolus begin to reaggregate forming a spherical structure, the "endobody" (Figs. 8A and 8B; Fig. 6, S5–S11). The endobody increases in diameter, from 1.8 μ in S5 to 4.8 μ in S11. It disappears during S12. From stages 5 through 9 the endobody is a densely packed fibrillar sphere with rough edges (Fig. 8B). However, by S10A the border of the endobody becomes smooth. The S10B endobody develops a cavity (*ca.* 5 μ^3) which approximately doubles in volume by S11 (see Fig. 6, S11). The endobody volume gradually increases from S5 until a maximum is reached by S10A (see Fig. 7). The volume then remains essentially constant through S10B and S11. Therefore, the increases in outer diameter seen in the S10B and S11 endobody are the result of the growth of the internal cavity rather than an increase in endobody material. From S5 through early S11 the endobody is in contact with the karyosome, but in late S11 this association is lost. The endobody stains with azure B in the characteristic manner of nucleolar material.

Small "packets" (*ca.* 600 Å diameter) of fibrillar material are seen in the oocyte nucleus from S4 through S9 (Fig. 6, S5-S9). At first this material is widely distributed, but as development proceeds these small clumps congregate in the area adjacent to the endobody. After S9 only a very small amount of the packet material is seen, and it is widely distributed throughout the nucleus. Figures 8A and 8B show the relationship between the endobody and packets in the S8 oocyte. The similar appearance of the material of the endobody and the packets and the

Cell 1 is the oocyte and cells 2–16 are nurse cells. The number in each canal specifies the division at which it was formed. The cystocytes are numbered so that only cells 1 and 2 possess a canal formed at the first division; cells 3 and 4 possess canals formed at the second and following divisions; cells 5–8 possess canals formed at the third and fourth divisions, and cells 9–16 possess canals formed only at the last division.

observed rough surface of the endobody suggests that there is movement of the packets either into or out of the endobody. By S10A the surface is smooth, and packets are no longer seen in the vicinity. We favor the movement of packets into

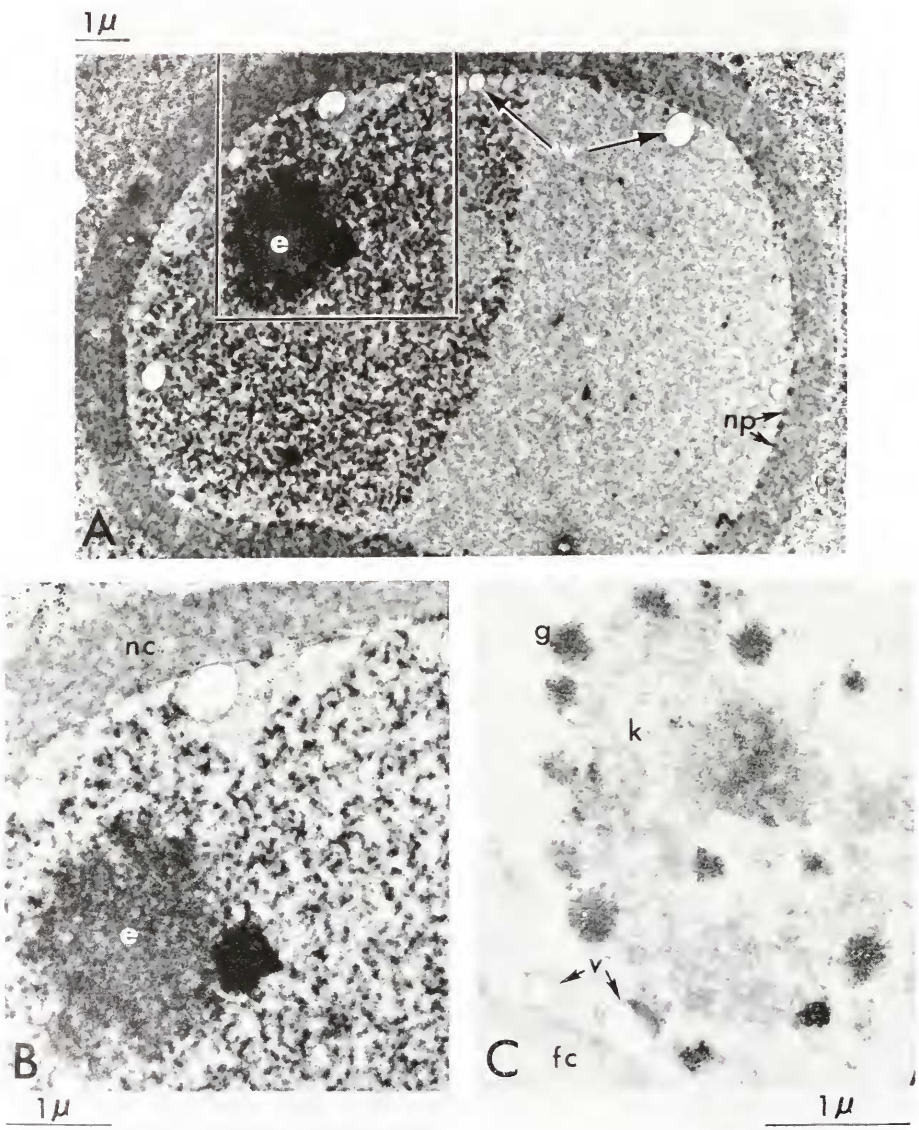


FIGURE 8. (A.) A section through a S8 oocyte which does not include the karyosome. A dense endobody (e) is seen surrounded by packets of nucleolar material. Vesicles (v) are present inside the oocyte nucleus, and nuclear pores (np) are evident; (B.) an enlargement of A, showing the rough border of the endobody and the 600 Å packets. The nuclear coat (nc) contains numerous dense areas; (C.) the karyosome (k) of another S8 oocyte contains clumps of granular (g) material.

the endobody because (1) as development proceeds, the packets get closer together and are found nearer the endobody, and (2) the endobody seems to grow at the expense of the packets, since a maximum endobody volume is seen at S10A, just

TABLE I

The number of oocyte and nurse nuclei traced at the light level and the nuclei photographed at the EM level. The "near" nurse nuclei are those in cells that are directly connected to the oocyte by ring canals, while the "distant" nurse nuclei are in cells separated from the oocyte by at least one other nurse cell. "N," the number of nuclei sectioned; "S," the average number of sections through each nucleus; (−) no nuclear halo visible; (+) to (+++++) indicate the relative amounts of nuclear coating visible

		oocyte nuclei		near nurse nuclei		distant nurse nuclei	
		N	S	N	S	N	S
stage 1	lt.	1 (−)	4	4 (−)	4	11 (−)	4
	em.	1 (−)	6	4 (−)	6	11 (−)	5
stage 2	lt.	2 (−)	5	12 (−)	5	32 (−)	5
	em.	1 (−)	3	2 (−)	10	8 (−)	7
stage 3	lt.	2 (−)	6	9 (−)	6	23 (−)	6
	em.	3 (+)	2	5 (−)	2	1 (−)	1
stage 4	lt.	3 (−)	6	18 (−)	6	38 (−)	6
	em.	2 (++)	5	3 (−)	3	5 (−)	3
stage 5	lt.	7 (+)	5	28 (−)	7	79 (−)	6
	em.	2 (+++)	4	4 (−)	2	7 (−)	4
stage 6	lt.	4 (++)	5	15 (−)	9	38 (−)	7
	em.	3 (++++)	8	3 (−)	2	6 (−)	6
stage 7	lt.	2 (+++)	7	10 (−)	12	22 (−)	10
	em.	1 (+++++)	12				
stage 8	lt.	5 (++++)	8	12 (−)	14	31 (−)	12
	em.	2 (+++++)	8				
stage 9	lt.	2 (+++++)	12	8 (−)	26	22 (−)	17
	em.						
stage 10A	lt.	1 (++++)	13	4 (−)	29	11 (−)	18
	em.	1 (++++)	3	1 (−)	1		
stage 10B	lt.	1 (+++)	18	4 (−)	33	11 (−)	24
	em.						
stage 11	lt.	2 (++)	26	8 (−)	35	22 (−)	30
	em.	1 (++)	5				
stage 12	lt.	1 (−)	6	Degenerate			
	em.	1 (−)	4				

after packets disappear; and (3) the volume of the endobody remains constant, once the packets are gone.

Small granules (about 400 Å in diameter) are associated with the karyosome of *Drosophila virilis*. These granules appear in aggregates of various sizes up to 0.3 μ in diameter. Although the amounts of this material are difficult to quantitate, it is very evident that there is an increase with time, and the maximum is reached at S10A. Cytochemical test with azure B (pH 4) indicate that these granules contain RNA.

Discussion

The general features of oogenesis in *Drosophila virilis* are very similar to those of *Drosophila melanogaster*. For example, the stage designations which were developed for *D. melanogaster* by King, Rubinson and Smith (1956) are applicable to *D. virilis* with little or no modification. As we will show in a subsequent paper, the relative times spent in events such as vitellogenesis, the follicle cell migrations, and the deposition of protective membranes are quite similar in the two species. In addition, the general developmental behavior of the nurse cell chromatin and nucleolar material is almost identical in the two species (see Cummings and King, 1969; King 1970 for *D. melanogaster*).

Koch, Smith and King (1967) demonstrated in *Drosophila melanogaster* that both cystocytes with four ring canals formed synaptonemal complexes, while the other 14 sister cystocytes did not. They also showed that, as it enters the vitellarium, one of the pro-oocytes switches to the developmental pathway being followed by the 14 nurse cells. The same developmental sequence was found in *D. virilis*. The synaptonemal complexes disappear from the oocyte nucleus by S4, and, as in *D. melanogaster* (Smith and King, 1968), by S5 the chromatin is incorporated into a compact karyosome. The small granules (about 400 Å in diameter) associated with the karyosome in *D. virilis* have also been seen in *D. melanogaster* and *D. immigrans* (Mahowald and Tiefert, 1970). We have determined that the number of granules reaches a maximum in S10A, a stage in which Mahowald and Tiefert have demonstrated RNA synthesis in the karyosome of *D. melanogaster*.

The behaviors of the oocyte nucleoli of *D. virilis*, *D. melanogaster*, and *D. immigrans* are quite similar during previtellogenic stages. In all three species the granular elements of the nucleoli are lost by S3 (see Mahowald and Tiefert, 1970, for *D. melanogaster* and *D. immigrans*). The fibrillar elements then seem to reaggregate forming a spherical endobody. Bier, Kunz and Ribbert (1967) first described this structure in the nuclei of oocytes from a variety of insect species characterized by both panoistic and meroistic ovarioles (the genera *Blatella*, *Gryllus*, *Carabus*, *Musca*, *Calliphora*, *Pterostichus* and *Dytiscus* were represented). They found that the structure does not take up H^3-uridine in short term experiments, but does take up H^3-amino acids. Differences were seen in the size, shape and longevity of the endobody depending on the species. Even in the genus *Drosophila*, variations are evident. While the endobody in *D. melanogaster* and *D. immigrans* disappears by the onset of vitellogenesis (Mahowald and Tiefert, 1970), the *D. virilis* endobody remains until the beginning of metaphase I in S12. Both *D. melanogaster* and *D. immigrans* have the 600 Å packets of densely-staining material

which we have described and followed in *D. virilis.* Our evidence suggests that most of the packets are incorporated into the *D. virilis* endobody by S10A.

Sonnenblick (1950) made a light microscopic investigation of the meiotic divisions in recently laid eggs of *Drosophila melanogaster.* According to his account no centrioles or asters were present in the maturation division figures. *Drosophila virilis* oocytes appear to complete meiotic prophase earlier than do *D. melanogaster* oocytes. Meiotic metaphase takes place during S13 and S14 in *D. virilis,* also in the absence of centrioles and asters! The polar areas of the spindles contain diffuse filamentous material and some accumulations of membranes, much like those described in the meiotic divisions of mammalian oocytes (Zamboni, 1970; Szollosi, 1972; Calarco, Donahue and Szollosi, 1972). Centrioles are found within germarial cystocytes and within nurse cells and oocytes of egg chambers in the vitellarium (Koch and King, 1969; Mahowald and Strassheim, 1970). As development proceeds the number of centrioles per oocyte increases and so does the number of nurse cells without centrioles. These findings suggest that after the last cystocyte division the centrioles detach from the cell membrane, that those within nurse cells are carried through the canals in the protoplasmic stream, and that eventually almost all of these centrioles are transferred to the oocyte. The population of centrioles presumably degenerates, since they do not seem to be required for the meiotic divisions.

Ring canals have been observed during the gametogenesis of organisms as diverse as annelids and mammals (see Cassidy and King, 1969; Ruby, Dyer, Gasser, and Skalko, 1970; Dym and Fawcett, 1971; King and Akai, 1971a; Moens and Go, 1972). The canals are found between sister gametocytes during both spermatogenesis and oogenesis. In those insects possessing polytrophic meroistic ovarioles, all but one of the sister germ cells subsequently function as nurse cells, but in many higher mammals the morphological distinction between nurse cells and oocytes is not obvious. However, Davidson (1968), noting that the majority of early oocytes degenerate in mammals, has proposed that some of the interconnected germ cells may function as trophocytes.

There is a general structural similarity in ring canals despite the wide phylogenetic separation of the organisms in which they occur. In all cases, the canal is seen to be round or oval in horizontal section, to be attached to the plasmalemma, and to have a thickened, densely staining rim. The specific features of canal morphology have been studied in detail in only a few organisms, but there is reason to believe that several fine structural aspects may be common at least among some insect orders. The great similarity between the canals of a hymenopteran, *Habrobracon juglandis* and a dipteran, *Drosophila virilis,* is seen when comparisons are made of leaves, vertical microtubules, and membrane connections. These two species are separated enough phylogenetically so that we anticipate finding these same fine structural features in other insect species.

During oogenesis the ring canals allow passage of materials from the nurse cells to the oocyte. Many of the long-lived mRNAs, and ribosomes needed during embryogenesis are provided at an earlier stage by the endopolyploid nurse cells. After fertilization, chromosomes are engaged in rapid mitoses and are inactive as far as transcription is concerned (Fan and Penman, 1970). King and Burnett (1959), Zalokar (1960), and Bier (1963) have demonstrated both the synthesis of

TABLE II

The final number of cystocytes per cluster during gametogenesis

Species	Sex	N	Reference
Drosophila hydei	Male	8	Hess and Meyer, 1968
Drosophila melanogaster	Male	16	Hess and Meyer, 1968
	Female	16	King, Rubinson and Smith, 1956
Habrobracon juglandis	Female	32	Cassidy and King, 1972
Bombyx mori	Male	64	King and Akai, 1971b
	Female	8	King and Akai, 1971b

RNA by nurse cells and its transport into the oocyte. The nurse cells are also major suppliers of mitochondria, lipid droplets, and low molecular weight materials (King, 1960; Cummings and King, 1970).

In the germaria of *Drosophila melanogaster* mitotic figures are found to be grouped into clusters of 2, 4, or 8 (Bucher, 1957; Grell, 1967; Johnson and King, 1972), and this observation demonstrates that interconnected cystocytes divide simultaneously. In *Xenopus laevis* somatic cell nuclei can be induced to undergo DNA replication by exposing them to egg cytoplasm (Graham, Arms, and Gurdon, 1966). This finding demonstrates that the test cytoplasm contains factors that stimulate mitosis. Perhaps in *Drosophila* the system of canals between cystocytes allows passage of soluble "mitogens" which initiate division in all cells in a given cluster. Thus, sister cystocytes should show an "all or none" type of mitotic activity, and their number (N) after their final division should be given by $N = 2^M$, where M equals the number of consecutive mitoses preceding meiosis. The values of N observed in a number of higher insects (see Table II) follow the 2^M rule, and these observations support the hypothesis that the ring canal system insures the mitotic synchrony of sister cystocytes during both oogenesis and spermatogenesis.

Dividing cystocytes are rarely observed in fixed *Drosophila* germaria. The data of Johnson and King (1972) indicate that only one germarium in 25 contains such cells. However, in two instances dividing cystocytes have been encountered in electron microscopic studies. In the cytokinesis illustrated by Koch and King (1969, their Figs. 2 and 3) vesicles and small tubules are aligned along the division plane separating two sister cystocytes. The canal rim is embedded in the center of this plaque. In the division illustrated by Mahowald (1971, his Figs. 1 and 2) paired, convoluted membranes extend to the canal. The tissues were fixed differently in the two cases. Koch and King (1969) used chromate-OsO_4 and Mahowald, glutaraldehyde. Taken together these results provide some useful information concerning cystocyte cytokineses. We now interpret the vesiculated areas to represent recently synthesized portions of the plasmalemma (see Figs. 9B and 9C). Presumably these are stabilized by glutaraldehyde, but tend to fragment when exposed to the harsher fixative. However, the portion of the plasmalemma to which the contractile ring microfibrils are attached is stable enough to resist the chromate-OsO_4 fixation and is therefore retained in both cases. The concept that new membrane is synthesized immediately beyond the contractile ring during cleavage formation is supported by the studies of Selman and Perry (1970) and Bluemink (1971) on cleaving amphibian zygotes.

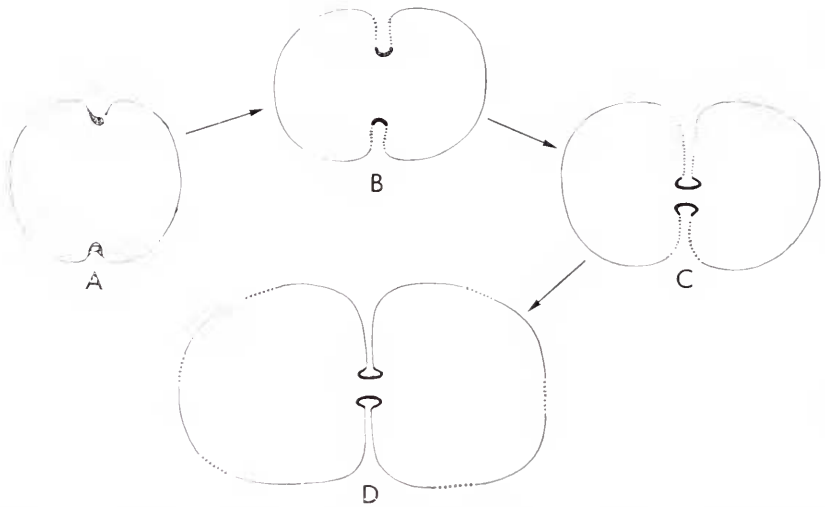


FIGURE 9. A diagrammatic representation of the proposed synthesis of new plasma membrane during the cleavage and subsequent growth of cystocytes. The contractile ring is represented by a thick solid line, old membrane by a thin solid line, and newly synthesized membrane by a stippled line. See text for further discussion.

Koch and King (1969) have shown in *Drosophila melanogaster* that, while the canal rims undergo morphogenetic changes, the average distance between ring canals remains relatively the same as the cystocytes grow. Therefore the plasmalemmal regions that bind microfibrils are not disturbed as the surfaces of the cystocytes expand. The above observations suggest that once cytokinesis is complete and fourth generation cystocytes start to grow, new plasma membrane is added to regions relatively distant from the canal rims (see Fig. 9D).

In the *Drosophila* male all cystocytes develop in an identical fashion, once divisions cease. In the female, however, the nurse cells and the oocyte follow different developmental pathways, although they are interconnected (see King, 1970 for details). Studies on the *female sterile* mutation provide clues to the mechanisms terminating cystocyte mitosis and initiating differentiation. Females homozygous for *fes* are sterile and produce "tumorous" ovarian chambers (King, Burnett and Staley, 1957, King, Koch and Cassens, 1961, Koch and King, 1964, King, 1969a). The mechanism which stops fourth generation cystocytes from further division in wild type *D. melanogaster* does not operate in the majority of *fes* chambers. In the mutant Johnson and King (1972) have shown that the normal arrested cleavage of cystocytes is disturbed so that divisions are often complete. They conclude that the product of the fes^{+} gene is required for the formation of a stable canal system, and they suggest that the product of the mutant gene is defective in this regard. In the abnormal cystocyte clusters found in *fes* an oocyte is produced only in those cells containing four canals (King, 1969b). If fewer canals are present, oocyte differentiation does not occur, regardless of the total number of cystocytes in the cluster. Johnson and King (1972) suggest that the signal that normally stops fourth generation cystocytes from further mitoses is the differentiation of pro-oocytes, and that these cells receive their cue to differentiate from the four canal rims that they alone possess.

We have presented cytological evidence suggesting that protein microfibrils continue to be deposited on the canal rims and that later masses of clustered microfibrils break off of each rim and are carried away in the cytoplasmic stream. The sizes of masses vary, but as one would expect the long and short axes of the masses are always parallel to the long and short axes of the constituent microfibrils. The masses seem to bind specifically to the oocyte nucleus and to form a coating that eventually reaches a thickness of a micron or more and is dense enough to exclude perinuclear mitochondria and ribosomes.

An interesting comparison can be made between the microfibrils associated with ring canals and the microfibrils making up the contractile rings found in cleaving animal cells (see Selman and Perry, 1970; Szollosi, 1970). Many investigators (see reviews by Rappaport, 1971; Tucker, 1971) believe that sliding interactions between the overlapping microfibrils generate the constrictive forces required for cleavage. Contractile ring microfibrils have a diameter of 30–70 Å and are always oriented circumferentially in the plane of the constriction. The microfibrils we see on the canal rims are of similar diameters and also have a circumferential orientation. We suggest that the ring canal rim is composed of old equatorial plasmalemma that retains an affinity for contractile ring microfibrils. However, we have no evidence for or against the argument that the microfibrils that attach subsequent to the arrested cleavage represent a heterogeneous assemblage of protein molecules that bind both to canal rims and to the surface of the oocyte nucleus only because these structures carry the appropriate receptors. For example, spindle microtubules are composed of fibrous protein subunits (see review by Adelman, Borisy, Shelanski, Weisenberg, and Taylor, 1968). These "tubulin" molecules, which have diameters ranging between 40 and 50 Å, should be abundant near canals and might contribute to the coatings. Regardless of the composition of the coatings, it is clear that the ring canal rims and the outer surface of the oocyte nucleus both tend to bind fibrous protein molecules and that this binding ability is shown by the rims before the nucleus. Perhaps the receptor molecules necessary for the subsequent binding of microfibrils pass from the rims to the oocyte nucleus; but this is but one of many plausible interpretations.

Studies on the mechanism of action of steroid hormones in birds and mammals (O'Malley, Sherman, and Toft, 1972; Steggles, Spelsberg, Glasser, and O'Malley, 1971) indicate that the cells of target tissues contain specific receptor proteins that bind to the hormones. The receptor protein-hormone complex then enters the nucleus, binds to the chromatin, and stimulates the transcription of mRNAs for the proteins whose synthesis is known to be induced by the hormones. In the *Drosophila* egg chamber the nucleus of the oocyte is much less active in terms of transcription than are those of the sister nurse cells. We assume that the nuclear coating prevents the transfer of high molecular weight materials to and from the oocyte nucleoplasm and thus helps to insulate the oocyte chromosomes from the influence of those compounds that stimulate transcription in the sister nurse cells.

The agent responsible for ring canal formation is probably a long-lived spindle apparatus (Fawcett, Ito and Slautterback, 1959; Brown and King, 1964) which arrests the advancing cleavage furrow and thus prevents separation of the sister cells. The portion of the spindle called the midbody remains in the bridge connecting the sister cells. Within the midbody, microtubules extending from the spindle poles overlap and are embedded in a dense matrix (Byers and Abramson,

1968). Buch and Tisdale (1962) report that the longevity of the midbody varies according to the species and the cell type in which it is found. Although the exact nature of ring canal formation is not understood, there is evidence that the advancing edge of the furrow may actually interact with the tubules making up the periphery of the midbody. This view is based on the structure of *Habrobracon* and *Drosophila* canal rims where the tubules found embedded in the leaves have the same orientation as the midbody microtubules (Cassidy and King, 1969).

Studies on ciliate cleavage (Tucker, 1971) suggest that the process includes two phases; the first rapid, the second slow. The rapid phase effectively separates the dividing cells except for a small neck. In a much slower phase, the last small interconnection is severed. Tucker suggests that the final nipping off of the sister cells is due to an additional or different mechanism from that involved in ring constriction. The ring canals of cystocytes could result from failure of this final cleavage mechanism.

Two patterns of cell division have been studied by electron microscopists. The first, which is commonly observed in animal tissues, requires the formation of a plasmalemmal furrow (Fawcett, Ito, and Slautterback, 1959). The second, which is commonly observed in higher plants, involves the laying down of a plaque of interconnected vesicles and tubules along the plane of division and later the coalescence of nearby tubules to form continuous sheets of membrane which segregate the cytoplasm of the sister cells (Porter and Machado, 1960). Centrioles and asters are associated with the mitotic apparatus in the first type of cytokinesis (Stubblefield and Brinkley, 1967). Centrioles and asters are missing from the mitotic apparatus in the second type of cytokinesis (Pickett-Heaps, 1971). The *Drosophila* oocyte is generated by the division of a third generation cystocyte, and a conventional mitotic apparatus with centrioles and asters is employed. The subsequent meiotic division figure lacks centrioles and asters, and the cell does not cleave. Taken together, the above facts suggest that centrioles are not necessary for the formation of spindles and for the sister chromosomes to be separated in an orderly fashion. This function is performed by much less conspicuous organelles. Pickett-Heaps (1971) has referred to these as microtubule-organizing-centers (MTOCs). Rappaport (1971) has reviewed the evidence which demonstrates that the establishment of a cleavage furrow requires two asters working in concert. Perhaps the centrioles generate asters, and in the zone where microtubules from the two asters are confluent an equatorial ring of plasma membrane is modified in some way that facilitates the assembly of contractile ring microfibrils. In *Drosophila melanogaster* according to Sonnenblick (1950) the polar body nuclei are not extruded from the egg but remain peripherally disposed in an anterodorsal island of ooplasm where they eventually disintegrate. It follows that in the case of the oocyte, centrioles can be dispensed with, since furrowing of a conventional sort does not occur during the meiotic divisions.

The fertilizing sperm brings in a centriole (Sonnenblick, 1950), and this is the progenitor of those seen during embryogenesis. After fertilization twelve synchronous mitoses occur, and the nuclei generated form a blastema. At the next mitosis cytokinesis takes place, and a cellular blastoderm is formed. In their study of this event in *Drosophila montana* Fullilove and Jacobson (1971) concluded that the oolemma starts to form furrows at those regions where the microtubules of adjacent asters overlap (see their Fig. 16).

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Summary

In females of *Drosophila virilis*, germ cells, characterized by conventional division, differentiate into "cystocytes" that engage in a specific number of mitoses, each followed by incomplete cleavage. The result is a cluster of 16 cells joined by canals. The canal rims are made up of overlapping leaves similar to those already described for *Habrobracon juglandis*. The *virilis* canal rims become coated with protein microfibrils. Masses of these fibrils detach from the rims and are carried in a stream of cytoplasm to the oocyte. Clusters of microfibrils adhere to the oocyte nucleus which eventually develops a coating of amorphous material 1 μ thick. The nuclei of the 15 sister nurse cells never develop such a coating. We suggest that the nuclear coating prevents the transfer of large molecules to the oocyte nucleoplasm and so insulates oocyte chromosomes from the influence of those compounds that stimulate transcription in the sister nurse cells. Centrioles and asters take part in cystocyte divisions, but are absent from the meiotic division figures. We conclude that centrioles function to generate asters. These in turn facilitate the attachment to localized regions of the plasmalemma of contractile microfibrils that enable furrowing to take place. Less conspicuous "microtubule-organizing centers" organize the spindle which functions to distribute the chromosomes during mitosis.

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